TITLE

In Vitro System for Replication of RNA-Dependent RNA Polymerase (RDRP) Viruses

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/265,437, filed January 31, 2001, the contents of which are herein incorporated by reference in their entirety.

FIELD OF THE INVENTION

This invention is directed toward the pharmaceutical and molecular biology arts, more particularly this invention is an *in vitro* system for the replication of the viral genomes of viruses that depend upon the enzyme RNA-dependent RNA polymerase (RDRP) for replication. The method of the invention provides an efficient means for measuring genomic replication in RDRP viruses, and also for the rapid screening of compounds for their ability to inhibit genomic replication of RDRP viruses, including the Hepatitis C virus (HCV).

BACKGROUND OF THE INVENTION

It is known that viral genomes can be made of DNA or RNA and can be double-stranded or single-stranded. Typically, viral genomes encode viral coat proteins that serve to package the genome after replication, and also nonstructural proteins that facilitate enzymatic replication of the viral genome in conjunction with cellular enzymes. In the case of some viruses having a single-stranded RNA genome, one of the nonstructural proteins encoded by the viral genome is RNA-dependent RNA polymerase (RDRP), which is needed by the virus to replicate its genomic sequence.

The viral enzyme RNA-dependent RNA polymerase is also called RNA replicase.

The viral family *Flaviviridae* is one such type of virus which is dependent upon its own RNA-dependent RNA polymerase in order to replicate. *Flaviviridae* is a family of viruses having a single-stranded RNA genome in the (+)orientation. The term "(+)orientation" is a convention used to designate single-stranded nucleic acid molecules which exist in the coding or sense orientation when read from the 5' to 3' direction. The *Flaviviridae* family comprises the flaviviruses, the animal pathogenic pestiviruses, the recently characterized GB viruses (GBV-A, GBV-B and GBV-C/hepatitis G), and most importantly from a human disease perspective, the genus *Hepacivirus* or Hepatitis C virus (HCV). The RNA genome of these viruses typically includes a single long open reading frame encoding a polyprotein that is proteolyically cleaved into a set of distinct structural and nonstructural protein products. Translation of the open reading frame of the genome is directed via a 5' untranslated region (UTR) which functions as an internal ribosomal entry site (IRES). The 3' end of the genome in these viruses comprises a highly conserved UTR region of variable length which is thought to be essential for replication.

The most well-known member of the *Flaviviridae* family of viruses is the Hepatitis C virus ("HCV"), which is a parenterally transmitted, hepatotropic virus that in primates causes acute and chronic hepatitis, as well as hepatocellular carcinoma. Approximately 2% of the world's human population is thought to be afflicted with HCV infections. No vaccine for HCV is currently available, and present treatment is generally limited to interferon monotherapy, or the combination of alpha-interferon with the

nucleoside analog ribavirin. (1)(2)(3)(4)(5).

HCV is a positive-stranded RNA virus having a genome 9.6 kb long comprised of a single, uninterrupted open reading frame encoding a polyprotein of about 3000-3011 amino acids. The HCV polyprotein is a precursor to the individual HCV proteins necessary for replication, packaging and infectivity. The structural region of the polyprotein precursor (including the C, E1, E2 and p7 proteins) is processed by host cell signal peptidases. The nonstructural region of the precursor (including the NS2, NS3, NS4A, NS4B, NS5A and NS5B proteins) is processed between NS2 and NS3 by NS2-3 protease, while processing in the NS3-NS5B region of the polyprotein is accomplished by NS3 protease activity. (6)(7)(8)(9)(10).

The mode of replication of the HCV virus is still speculative and current understanding is based upon analogy with other of the flavi-and pestiviruses. It is believed that HCV replication begins by viral penetration of the host cell and liberation of the viral genomic (+)single-stranded RNA from the virus particle into the cytoplasm of the cell. The viral RNA is translated by cellular enzymes, and the encoded viral polyprotein is processed into several distinct functional viral proteins including RNA-dependent RNA polymerase protein (RDRP). RDRP then proceeds to synthesize (-)stranded RNA intermediates (from template viral genomes) which in turn serve as templates for synthesis of new (+)stranded RNA molecules. These (+)stranded viral RNA molecules can then be used for further viral polyprotein expression, for synthesis of new (-)stranded RNA molecules, or for packaging into progeny virions which can then be released from the infected cell to spread the HCV infection. (1).

Presently, there are no efficient systems for *in vitro*

monitoring of the replication of the RDRP viruses of the *Flaviviridae* family. As a result, there is a lack of means for studying the mechanism of replication of these (+)stranded RNA viruses, or for determining the ability of a compound or condition to inhibit such replication. While cell-based systems for HCV replication have been described (11), these systems rely on protocols and endpoints that are not easily formatted into platforms for screening large numbers of compounds for anti-viral activity (12). The present invention provides a solution to these problems by providing a system for the efficient *in vitro* manipulation and monitoring of the replication of RDRP viruses. The system of the invention can be assembled so as to provide a convenient platform for screening inhibitors to RDRP viral replication. The method of the invention also provides a means to design therapies for the *in vivo* treatment of cells that are infected with RDRP viruses.

SUMMARY OF THE INVENTION

The present invention provides an efficient *in vitro* method for measuring the replication of the genome of viruses that are dependent upon RNA-dependent RNA polymerase for replication (these types of viruses are herein referred to as "RDRP viruses"). The method comprises the steps of culturing virally-compatible eukaryotic cells, which have been transfected with the cDNA of the genome of the RDRP virus, and transfecting these cultured cells with a construct of the invention, which construct comprises the cDNA, in antisense orientation, of a reporter gene sequence. The reporter gene cDNA sequence of the construct is operably linked on its 5′ end with the cDNA of the untranslated region (hereinafter "UTR") in antisense orientation of the native 3′ end of said RDRP virus, and is operably linked on

its 3′ end with the cDNA of the UTR in antisense orientation of the native 5′ end of said RDRP virus. Thus, the construct will be comprised of the cDNA, in antisense orientation, of a reporter gene flanked by the 3′ and 5′ UTRs of the native RDRP viral genome. Transfected cells containing the construct of the invention are cultured for a sufficient period of time under conditions which are permissive for replication of said RDRP virus, and the cells are analyzed for the presence of the protein encoded by the reporter gene. If the cDNA of the RDRP viral genome has been replicated and processed within the cultured cell, viral RDRP enzyme will have been synthesized, thereby enabling polymerization of the construct and synthesis of the protein encoded by the reporter gene. Thus, detection of the reporter protein in the cells provides a means to monitor and measure the genomic replication of said RDRP virus.

In another aspect, the invention provides an efficient *in vitro* method for identifying compounds or conditions which inhibit the genomic replication of viruses that are dependent for replication on RNA-dependent RNA polymerase (an RDRP virus). The method comprises the steps of culturing virally-compatible eukaryotic cells, which have been transfected with the cDNA of all or a portion of the genomic sequence of the RDRP virus, and transfecting these cultured cells with a construct of the invention, which comprises the cDNA in antisense orientation of a reporter gene sequence. The reporter gene cDNA sequence is operably linked on its 5′ end with the cDNA of the untranslated region (UTR), in antisense orientation, from the native 3′ end of said RDRP virus, and is operably linked on its 3′ end with the UTR, in antisense orientation, from the native 5′ end of the RDRP virus. The cultured cells are exposed to a

compound or condition suspected of being capable of inhibiting the genomic replication of the RDRP virus, and thereafter or concurrently the cells are cultured for a period of time under conditions which are permissive for genomic replication of the RDRP virus. The cells are analyzed for the presence of the protein encoded by the reporter gene sequence, whereby a decrease in the level of the reporter protein indicates that the suspected compound or condition is capable of inhibiting genomic replication of the RDRP virus.

The present invention also provides a method of selectively affecting a cell which is infected with a virus that is dependent for genomic replication upon RNA-dependent RNA polymerase (an RDRP virus). The method comprises transfecting tissues, or cells which are infected with an RDRP virus, with a construct of the invention comprising the cDNA in antisense orientation of a gene or sequence which encodes a protein that is capable of affecting the cell, wherein the cDNA sequence encoding said protein is operably linked on its 5′ end with the cDNA of the untranslated region (UTR), in antisense orientation, of the native 3′ end of said RDRP virus and is operably linked on its 3′ end with the cDNA of the UTR, in antisense orientation, of the native 5′ end of said RDRP virus. Sufficient time for genomic replication of said RDRP virus is allowed. Thus, upon genomic replication of the RDRP virus, RNA-dependent RNA polymerase (RDRP) is produced which will cause polymerization of the construct thereby allowing synthesis within infected cells of the affecting protein. In this manner, only cells that are infected with the RDRP virus will be affected, thereby affording a mechanism to selectively affect RDRP virally infected cells within a mixed population of infected and normal cells. In a

preferred aspect of this embodiment of the invention, the effect achieved is to selectively harm or kill cells which are infected with the RDRP virus by inserting into the construct the cDNA of a sequence encoding a protein which is harmful or fatal to the cell.

In all aspects of the present method of the invention, a preferred embodiment includes wherein the RDRP virus is selected from the viral family *Flaviviridae*. It is most preferred that the RDRP virus is HCV.

A further preferred embodiment in all aspects of the method of the invention includes wherein the construct of the invention further comprises the cDNA of a delta ribozyme sequence, in sense orientation, operably linked to the 3' end of the construct adjacent to the 3' end. When the RDRP virus is HCV, the cDNA of hepatitis delta ribozyme, in sense orientation, is operably linked to the 3' end of the cDNA of the 5' UTR of the native HCV viral genome.

In another aspect, the invention provides a construct comprising the cDNA, in antisense orientation, of a reporter gene sequence wherein said reporter gene cDNA sequence is operably linked on its 5' end with the cDNA of the UTR, in antisense orientation, of the native 3' end of an RDRP virus and is operably linked on its 3' end with the cDNA of the UTR, in antisense orientation, of the native 5' end of the RDRP virus. Alternatively, in another aspect of the invention, instead of the antisense cDNA of a reporter gene sequence, a construct may comprise the antisense cDNA of an "affecting gene" wherein said gene encodes a protein which is capable of affecting the cell, preferably harming or killing the cell. In these aspects of the invention it is preferred that the RDRP virus is HCV.

The constructs of the invention further comprise an operably linked constitutive or inducible promoter.

It is also preferred that the constructs of the invention further comprise the cDNA, in sense orientation, of the hepatitis delta ribozyme operably linked to the 3' end of the cDNA, in antisense orientation, of the 5' UTR of the native viral genome.

And in another aspect, the invention provides a eukaryotic cell which has been transfected with a construct of the invention, preferably a primate cell, most preferably, a human cell.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Schematic for production of RDRP-dependent luciferase activity in the 293B4α cell line.

Figure 2. Cloning strategy for the construction of pMJ050.

Figure 3. Nucleotide sequence of pMJ050, presented from left to right in 5' to 3' orientation, Fig. 3A. showing the nucleotides comprising the SV40 promoter and the HCV 3'UTR (in antisense orientation); Fig 3B. showing the luciferase coding region (in antisense orientation); the HCV 5' UTR sequence (in antisense orientation); and the hepatitis delta virus ribozyme sequence (in sense orientation); and Fig 3C. showing the plasmid backbone sequence.

Figure 4. Production of luciferase in 293FL#9 cells stably transfected with pMJ050.

Figure 5. Production of luciferase, HCV core, HCV serine protease, and HCV RDRP in the 293B4α cell line.

Figure 6. Production of luciferase sense and antisense RNA in the 293B4α cell line.

Figure 7. Schematic representation of the mechanism of the invention in a B4alpha human kidney cell which has been transfected with the genome of HCV,

using luciferase as the reporter gene in a construct of the invention.

DETAILED DESCRIPTION OF THE INVENTION

A convenient *in vitro* system that models viral replication for several members of the important viral family *Flaviviridae* has not been described to date. This lack of an *in vitro* system has significantly hindered research in this field directed towards development of antiviral agents for the treatment of viral infection, particularly for HCV infection. Described herein is an *in vitro* system that can be formatted to allow detection of cells in which RDRP genomic replication is occurring. The method employs a construct that expresses a detectable reporter protein in response to RDRP viral genomic replication. The method of the invention can be manipulated to screen for compounds or conditions having the ability to inhibit RDRP viral genomic replication. The method also provides a mechanism in which RDRP virally-infected cells can be selectively affected.

Various definitions and abbreviations are provided throughout this document. Most words, unless otherwise defined, have the meaning that would be attributed to those words by one skilled in the art of the invention.

The following abbreviations are used throughout this application: HCV: Hepatitis C virus; DNA: deoxyribonucleic acid; RNA: ribonucleic acid; UTR: untranslated region; hdvribo: hepatitis delta virus ribozyme; PCR: polymerase chain reaction; RDRP: RNA-dependent RNA polymerase enzyme; IRES: internal ribosome entry site; RT: reverse transcriptase; and RT-PCR: reverse transcription polymerase chain reaction.

As used herein the term "*in vitro*" means occurring

outside of a living organism; in contrast to the term "*in vivo*", which means occurring within a living organism. *In vitro* can describe processes and conditions occurring within cultured cells, or occurring within cellular lysate systems that contain the cellular components necessary to perform the process in question.

Applicants contemplate that the *in vitro* methods of the invention relating to measuring RDRP genomic replication and identifying inhibitors of such replication can be conducted in cell culture systems, or alternatively, in the cellular lysate systems of virally compatible eukaryotic cells.

Within the context of this invention, the term "virally-compatible cells" refers to eukaryotic cells that contain the necessary cellular proteins required by the RDRP virus to complete replication of the virus genome. Virally-compatible cells include, but are not limited to, cells in which the viral particle is able to complete its entire replication cycle i.e., the virus is able to reproduce and generate other infectious viral particles. Also included are cells that may not be able to sustain the entire viral replication cycle, but which are able to sustain the replication of the viral RNA genome. Examples of preferred virally-compatible cells include mammalian, especially human, liver and kidney cells, and B and T cells.

"Virally-compatible cells which have been transfected with the cDNA of the genomic sequence of an RDRP virus" refers to virally-compatible cells into which have been stably incorporated a functional genomic sequence of the virus under study. When the method is conducted in order to study and measure replication of the viral genome, it will be preferable to incorporate most or all of the native viral genomic sequence, in order to most effectively mimic and study native replication. When the method is

conducted in order to identify inhibitors of viral replication, it is possible to incorporate into the cellular genome all of the genome, or alternatively only those selective portions of the viral genome which encode proteins to be studied, so long as the selected portion of the viral genome includes the sequence that encodes the RNA-dependent RNA polymerase, which is known as the NS5B portion of the HCV genome. Methods for stably transfecting all or selective portions of the viral genome into suitable cell lines are known by those skilled in the art. For example, such methods are reported in "Continuous Human Cell Lines Inducibly Expressing Hepatitus C Virus Structural and Nonstructural Proteins," Darius Marpour, Petra Kary, Charles M. Rice and Huber E. Blum (1998) Hepatology 28:192201. "Transfection of a Differentiated Human Hepatoma Cell Line (Huh7) with In Vitro-Transcribed Hepatitis C Virus (HCV) RNA and Establishment of a Long-Term Culture Persistently Infected with HCV," Young J. Yoo et al, J. of Virology, Vol 69, No.1, Jan 1995, p. 32-38. Genomic sequences for the flaviviruses are generally available in the scientific literature, for example, see www.ncbi.nlm.nih.gov/genbank for the Genbank library of sequences that includes viral and the *flavivirus* gene sequences.

Applicants contemplate that the *in vitro* methods of the invention relating to measuring genomic replication of RDRP cells and identifying inhibitors of such replication can be conducted not only in cell culture systems of virally-compatible cells, but also in cell lysate systems of those cells. For example, cells from HCV-infected cell culture or tissues removed from an HCV-positive individual can be used to create a cell lysate that can serve as a source of the HCV replicative proteins. This lysate can be prepared by lysing the infected culture or tissue cells by methods well

known in these art, for example, by chemical or physical means, and clarifying the cell lysate of macromolecule cellular debris by centrifugation. Reporter RNA produced by *in vitro* transcription of the reporter constructs of the invention can be added to the cell lysate described above and the lysate with added reporter RNA can be incubated under proper conditions permissive for genomic replication. Such conditions (e.g., temperature, pH, salt concentrations, etc.) are known or can be readily determined experimentally by those skilled in the art for the particular system selected for the assay. Lysates are then assayed to see if the reporter protein has been produced, thereby indicating that viral genomic replication has occurred within the lysate system. Such lysate systems are amenable to rapid, high throughput screening for inhibitors of RDRP viral replication.

The term "replication" as used within the disclosure herein regarding viruses relates to the replication of the genome of the virus, rather than whole virus replication which results in an infectious particle.

The term "transfecting" as used herein refers to the process of inserting heterologous DNA into a eukaryotic cell by chemical, physical or other means that include but are not limited to liposomal transfer, in which liposomal micelles containing the heterologous DNA transfer the DNA into the cell by fusion with the cell membrane; $CaPO_4$ or DEAE-dextran shock, in which these chemical moieties physically disrupt the cell membrane allowing macromolecules to pass from the outside to the inside of the cell; and electroporation, in which electrical shock is used to disrupt the cell membrane allowing macromolecules to pass from the outside to the inside of the cell. Such methods are well known in these arts. Newly emerging nucleic acid

delivery systems include the adenoviral and adeno-associated viral systems, which are being developed and used to deliver heterologous DNA sequences into human tissues for the purposes of gene therapy. Also, as used herein, the term "transfected" includes both stably transfected cells, in which the transfected DNA recombines with the host cell DNA such that it becomes a permanent part of the genome of the host cell, and also transiently transfected cells, in which the transfected DNA remains independent of the host cell DNA and is either destroyed by host cell mechanisms which act to defend the cell from "infection" with heterologous DNA or is diluted out by the replication of the host cell.

Moreover, the RNA which is used as the template for replication can be delivered to the cell by methods including but not limited to virus infection, transfection of *in vitro* transcribed RNA, and transcription of DNA that is stably or transiently transfected into the host cell. RNA transcription from stably or transiently transfected heterologous DNA can occur either constitutively or inducibly.

Within the context of the invention, those skilled in this art will understand that transcription of transfected DNA will be driven by an operably linked promoter system. A "promoter" is a regulatory nucleic acid sequence that is capable of controlling the expression of a coding sequence or functional RNA. In general, a coding sequence will be located 3' to the promoter sequence. Promoters may be derived in their entirety from a native gene, or be comprised of different elements derived from different promoters found in nature. It is understood that various well known promoters are suitable to direct expression of any number of different coding sequences depending on cell and tissue type, in response to different stimuli, or at

different stages of cellular or tissue development. Furthermore, the promoter sequence, which is part of the transfected DNA of the invention, will determine if expression of the transfected DNA will be constitutive or inducible. Examples of constitutive promoters include but are not limited to the cytomegalovirus immediate-early promoter, the SV40 viral promoter, human immunodeficiency virus long terminal repeat promoter, and the chicken beta-actin promoter. Examples of inducible promoters include but are not limited to the tetracycline-responsive promoter, the ecdysone-inducible promoter, and the mifepristone-inducible promoter.

The term "operably linked" refers to the association of two or more nucleic acid sequences on a single nucleic acid fragment so that the function of one is affected by the other. For example, a promoter is operably linked with a coding sequence when it is capable of affecting the expression of that coding sequence (i.e., the coding sequence is under the transcriptional control of the promoter). Coding sequences can be operably linked to regulatory sequences such as promoters in the sense or antisense orientation.

The term "RNA-dependent RNA polymerase virus" or "RDRP virus" means a virus which is dependent upon a functional RNA-dependent RNA polymerase for the replication of its nucleic acid genome and the production of infectious virus. In particular relating to the present invention, we include viruses that are members of the *Flaviviridae* family. It has been shown that the replication of all of the members of this virus family are dependent upon the virus's RNA-dependent RNA polymerase for replication of the virus genome. The RDRP performs several essential steps in the replication of RNA including the interaction of the RDRP

with the 3' and 5' untranslated regions (UTR) of the genome, initiation of the synthesis of the new RNA strand, and continued elongation of the growing progeny RNA. The UTR's for each of the different members of this family are unique to that particular virus, and have been identified, sequenced and placed in the public domain (i.e.,Genebank data base), and are thus readily obtainable. In a preferred aspect of this invention the sequence for the ribozyme from the hepatitis delta virus is placed at the 3' end of the constructs of the invention. This sequence, when transcribed into RNA, has catalytic function and will cleave itself from the 3' end of the RNA transcript. When using HCV, for example, this cleavage event results in a proper 3' end for the HCV 3' UTR in the antisense RNA transcripts and a proper 5' end in the HCV 5'UTR in the sense RNA transcripts of this invention. The catalysis of the hepatitis delta virus ribozyme is regulated by sequences contained within the ribozyme itself. This being so, the sequence for the hepatitis delta ribozyme included in the description of this construct can be used with other RDRP virus systems (besides HCV) and accordingly in most or all of the antisense reporter constructs of the invention.

Within the context of the present invention, the term "expression" refers to the transcription of DNA resulting in the production of sense or antisense RNA, and may also refer to translation of mRNA into a polypeptide, such as the reporter protein.

The term "reporter gene" or "reporter gene sequence" refers to any gene encoding a protein which can be expressed and conveniently detected in a eukaryotic cell including chemically, spectrophotometrically, immunologically, colorimetrically, radioactively or through a receptor-mediated cascade system. Examples of reporters include but

are not limited to luciferase, secreted alkaline phosphatase, beta-galactosidase, hepatitis B virus surface antigen, herpes simplex virus thymidine kinase, genticin-resistance, zeocin-resistance, hygromycin-resistance, and puromycin-resistance.

A reporter gene sequence affects cells or lysates within the method of the invention by producing an effect that can be detected. Applicants contemplate that other gene sequences could be used in place of a reporter sequence when the goal of the researcher is to selectively produce another specific effect within cells wherein viral genomic replication is occurring. Thus, if a gene or genes resulting in a deleterious effect are selected for insertion into the construct in place of (or in addition to) the reporter sequence, the method of the invention offers a mechanism to design therapeutic methods for the selective treatment of cells infected with a flavivirus, wherein the construct containing an affecting gene or genes is delivered *in vivo* to cells known to be infected with a replicating flavivirus.

The term "construct" when referring to the "construct of the invention" as used herein refers to a DNA sequence which comprises a coding sequence for a reporter gene or an affecting gene, as the terms are used herein, plus regulatory sequences related to expression of that coding sequence including particularly promoters that facilitate transcription of the coding sequence and also including any 5' and/or 3' transcribed but untranslated sequences that are associated with the coding sequence and may be required, plus the 3' and 5' untranslated regions (UTRs) of the RDRP virus under study. These sequences may be in the sense or antisense orientation. The total construct sequence is created using standard molecular biology techniques. The

construct of the invention may also include an operably linked ribozyme sequence.

A representative construct of the invention is provided in Figure 1, and includes (from 5' to 3') an SV40 promoter sequence, in sense orientation, operably linked to the HCV 3'UTR, in antisense orientation, linked to a coding sequence for luciferase protein, in antisense orientation, linked to the HCV 5'UTR, in antisense orientation, linked to the coding sequence for the hepatitis delta ribozyme, in sense orientation, wherein said construct is delivered to the cell via the plasmid entitled pMJ050.

In one aspect of the invention, a method is provided for screening inhibitors of viral replication. Compounds and conditions that are potentially capable of inhibiting viral replication include but are not limited to small molecular weight synthetic chemicals, organic compounds that are derived from living or once living organisms, synthetic chemical compounds based on organic compounds derived from living or once living organisms, as well as various conditions including different frequencies of sound, and various wavelengths of light and temperature. By example, compounds may include small molecules, peptides, proteins, sugars, nucleotides or nucleic acids, and may be natural or synthetic.

The method of screening compounds for inhibitors of viral replication includes any protocol which utilizes cells or cell lysate containing all or a portion of a viral genome sufficient to express a functional RNA-dependent RNA polymerase, to which cell or lysate, is added the appropriate reporter construct of the invention. The viral genome and reporter construct system are placed in the presence of a potential inhibitor(s) of viral replication, under conditions amenable to replication of that viral

genome. Thus, compounds or conditions capable of inhibiting replication of the viral genome, and/or capable of inhibiting the functionality of the expressed RDRP enzyme, can be identified via inhibition of expression of the reporter sequence.

The methods of the invention are functional when enough of the viral genome is present in the system to result in production of functional RNA-dependent RNA polymerase. Thus, the coding sequence of the viral genome that is used in the cultured cells or cell lysate can contain the coding sequence of the RNA-dependent RNA polymerase as part of the entire viral genome, or alternatively, it can contain subgenomic fragments of the viral genome, encoding, for example in HCV, the NS2 to NS5b region, or from the NS3 to NS5b region. In light of this aspect, the methods of the invention permit screening for inhibitors which have the ability to inhibit not only the NS5b RDRP, but also the NS2 protease, NS3 protease, and NS3 helicase as well, individually or collectively. Specifically, the ability to screen compounds for the potential to inhibit many different targets allows for the testing of different combinations of inhibitors targeted at one or more of the essential enzyme functions establishing whether interaction between the compounds favorably or deleteriously effects the ability of the compounds to inhibit the replication of the RNA.

EXAMPLES

The following examples demonstrate the method of the invention, but should not be viewed as limiting of the scope of the invention. Based upon the present disclosure many possible variations of the method of the invention will become apparent to those skilled in these arts.

In Examples 1 through 3 examines HCV viral genomic

replication in human kidney cells, using firefly luciferase as a reporter gene within the construct of the invention. Example 3 demonstrates use of the method to confirm known inhibitors of HCV replication using the method of the invention. Example 4 demonstrates a semi high-throughput screening assay for inhibitors or HCV genomic replication.

Example 1

It is known that *Flaviviridae* viral replication takes place through a step catalyzed by the viral RNA-dependent RNA polymerase (RDRP), an enzyme not normally found in eukaryotic cells. A substrate for HCV RDRP was selected that consists of an antisense sequence of the firefly luciferase gene, a common reporter gene used in cell biology. To make this sequence appear "HCV-like" it was flanked with the 5' and 3' untranslated regions (UTR) of the native HCV viral genome in the same orientation as they are found in the (-)strand of the HCV replicative intermediates. Using the convention employed herein, the orientation existing in the (-)strand of the RNA genome will be referred to as the antisense orientation when read from the 5' to 3' direction. To demonstrate a preferred embodiment of the method, the hepatitis delta ribozyme hdvribo was attached to the 3' end of the HCV 5' UTR sequence, such that when the hdvribo processes the RNA, the sequence integrity of the 5' UTR would be maintained (the strategy for the reporter is shown in Fig. 1). This was done because it is known to those skilled in the art that the 5' UTR also acts as an internal ribosomal entry site (IRES) (13), and it was desirable to keep the 5' UTR sequence as true as possible to that found in the native virus.

This construct was stably transfected into a 293 cell line (human embryonic kidney cells) and designated 293 FL#9

(this cell line had been previously transfected to contain a full length cDNA copy of the native HCV genotype 1b genome). The cell line containing the construct of the invention, 293B4α, was demonstrated to produce active, detectable luciferase as a result of genomic replication of the HCV viral genome.

Preparation of the pMJ050 Construct

pMJ050 was prepared in three steps. First, the antisense sequences of the 3' untranslated region of the HCV genome (3'UTR) and the firefly luciferase gene were joined together; second, the antisense sequence of the HCV 5' untranslated region (5'UTR) and the sense sequence of the hdvribo were joined together; and finally these two constructs were joined together resulting in a sequence which consisted of the antisense sequences of the "3'UTR-firefly luciferase-5'UTR-hdvribo (in the sense orientation)", respectively as read from 5' to 3' (Fig. 2).

Construction of the 3'UTR and luciferase sequence

The antisense sequence of the HCV 3'UTR was PCR amplified from plasmid p90 (supplied by Dr. Charles Rice, Washington University at St. Louis) using PCR primers, 3'UTR5'(new) and 3'UTRHO (for the nucleotide sequences of all oligos, see Table 1). The antisense sequence of the firefly luciferase gene was PCR amplified from plasmid pGL3 (Promega Corporation, Madison WI) using PCR primers LUCHO and LUCIF3'. To join these two PCR products together, overlapping PCR was performed in which equimolar amounts of the two PCR products were mixed with oligos, 3'UTR5' and LUCIF3', and the DNA amplified by PCR.

Table 1

Nucleotide Sequences of Oligos Used to Create and Sequence pMJ050

OLIGO NAME/ SEQ ID Nos:	OLIGO SEQUENCE (Read 5' TO 3')
3'UTR5'new SEQ ID NO:1	GCG TTT AAG CTT ACA TGA TCT GCA GAG AGG
3'UTRHO SEQ ID NO:2	GGC GGA AAG ATC GCC GTG TAA AGG TTG GGG TAA ACA CTC CGG
5'UTR5' SEQ ID NO:3	CTG TGG ACG TCG GTT GGT GTT ACG TTT GGT TTT TCT TTG AGG TTT AGG
5'UTRHO SEQ ID NO:4	GGC TGG GAC CAT GCC GGC CGC CAG CCC CCT GAT GGG GGC
LUCHO SEQ ID NO:5	CCG GAG TGT TTA CCC CAA CCT TTA CAC GGC GAT CTT TCC GCC
LUCIF3' SEQ ID NO:6	TTG GTA GAC GTC CAA TGG AAG ACG CCA AAA TAA AGA AAG G
HEPHO SEQ ID NO:7	GCC CCC ATC AGG GGG CTG GCG GCC GGC ATG GTC CCA GCC
RIBOHD3' SEQ ID NO:8	CTC AAG CTC TAG AGA GAT TTG TGG GTC CC
LUCACA(+) SEQ ID NO:9	GAA GAC GCC AAA AAC ATA AAG AAG GGC CCG GCG CCA
LUCACA(-) SEQ ID NO:10	TGG CGC CGG GCC CTT CTT TAT GTT TTT GGC GTC TTC
UTRRNA(+) SEQ ID NO:11	CCT CTT AGG CCA TTT CCT GTT TTT TTT TTT
UTRRNA(-) SEQ ID NO:12	AAA AAA AAA AAC AGG AAA TGG CCT AAG AGG
LUCFOR SEQ ID NO:13	CCG AGT GTA GTA AAC ATT CC

LUCREV SEQ ID NO:14	CTC GCA TGC CAG AGA TCC
LITFOR SEQ ID NO:15	GAT CTT CGA ATG CAT CGC GCG C
LITREV SEQ ID NO:16	GGC CTT GAC TAG AGG GTA CC

The product of the overlapping PCR was digested with the restriction enzymes, *Hind* III and *Aat* II, and ligated into pLitmus28 (New England Biolabs, Beverley, MA) which had been linearized with *Hind* III and *Aat* II. The plasmid from the ligation reaction, pLitmus283'UTR luciferase was transformed into chemically competent *E. coli* DH5α cells. *E. coli* that had become transformed with this plasmid were selected by the ability to grow on solid nutrient agar containing ampicillin. Plasmid DNA was isolated from ampicillin-resistant bacterial cells and the sequence was verified by restriction enzyme analysis using *BsrG* I, *Hind* III and *Aat* II, and sequence analysis using sequencing oligos LUCFOR, LUCREV, LITFOR, and LITREV.

Construction of the Antisense 5'UTR
and Sense hdvribo Sequence

The sequence of the hepatitis delta virus ribozyme (hdvribo) was PCR amplified from plasmid pFullLengthVec (provided by Dr. William Mason, Fox Chase Cancer Center, Philadelphia, PA) using PCR primers HEPHO and RIBOHD3'. The 5'UTR of the HCV genome was PCR amplified from plasmid pSignalIRES (provided by Robert Kovelman, Signal Pharmaceuticals, San Diego, CA) and joined to the hdvribo sequence by overlapping PCR using PCR primers 5'UTR5', 5'UTRHO, and RIBOHD3'.

The DNA product from the overlapping PCR was digested

with the restriction enzymes, *Xba* I and *Aat* II, and ligated into pLitmus28 that had been linearized by digestion with *Xba* I and *Aat* II. The recombinant plasmid, pLitmus285'UTRΔribo was transformed into chemically competent *E. coli* DH5 α cells. *E. coli* that had been transformed with this plasmid were selected by the ability to grow on solid nutrient agar containing ampicillin. Plasmid DNA was isolated from ampicillin-resistant bacterial cells and the sequence was verified by restriction enzyme analysis with *Xba* I and *Aat* II, and sequence analysis using primers 5'UTR5' and RIBOHD3'.

Construction of pMJ050

The inserts in plasmids, pLitmus283'UTR luciferase and pLitmus285'UTR hdvribo were joined together by digesting both plasmids with restriction enzymes *Hind* III and *Aat* II. Equimolar amounts of DNA were mixed and ligated together. The DNA resulting from the ligation reaction was transformed into chemically competent *E. coli* DH5α cells. *E. coli* that had become transformed were selected by the ability to grow on solid nutrient agar containing ampicillin. Plasmid DNA was isolated from ampicillin-resistant bacterial cells. Insertion of the reporter gene was verified by restriction enzyme analysis using *Hind* III and *Xba* I.

For the reporter gene to be transcribed in an eukaryotic cell, the reporter gene from pLitmus28reporter had to be placed into a plasmid that contained an eukaryotic promoter. To accomplish this, the reporter gene was removed from pLitmus28reporter by restriction digestion with *Spe* I and *Xba* I and ligated into the plasmid pZeoSV that had been previously linearized by restriction digest with *Hind* III and *Spe* I. The DNA resulting from the ligation reaction was transformed into chemically competent *E. coli* DH5α. *E. coli*

that had become transformed were selected by the ability to grow on solid nutrient agar containing zeocin. Plasmid DNA was isolated from zeocin-resistant bacterial cells and the sequence of the recombinant plasmid was verified by restriction enzyme analysis using *Hind* III, *Tth*III I and *Kpn* I, and sequence analysis with oligos 3'UTR5'new, 3'UTRHO, 5'UTR5', 5'UTRHO, LUCHO, LUCIF3', HEPHO, RIBOHD3', LUCFOR, and LUCREV. The plasmid containing the correct sequence construct was designated pMJ050 (Fig. 3).

Example 2

Creation of the 293B4α Cell Line

(a) Transfection and selection of zeocin-resistant 293FL#9 cells.

To create a cell line the would express the antisense luciferase construct as RNA in the environment of the HCV proteins, pMJ050 was transfected into 293FL#9 cells by electroporation (3-10 μg of plasmid into 5 x 10^6 cells; one pulse at 960 °F and 0.2 kV in a BioRad electroporator). Transfectants were grown in the presence of 250 μg/ml each of G418 and zeocin for several weeks to select for cells that had stably integrated pMJ050 into their genome. Forty-eight zeocin-resistant stable transfectants were randomly selected and expanded further.

(b) Luciferase Assay

The 48 stable transfectants were tested for the ability to express active luciferase using the commercially available Luciferase Assay System (Promega Corp., Madison, WI) as directed by the manufacturer. Briefly, the 1 x 10^6 cells from each of the 48 clonal cell lines was lysed with 100 μl of Lysis Buffer. The lysates were clarified by centrifugation and stored at -80 °C. Twenty microliters of

cell lysate was assayed for luciferase activity by the addition of 100 μl of luciferin substrate and quantification on a luminometer.

Twelve of 48 resultant clones expressed various amounts of luciferase activity. These twelve were grown for several more weeks in the presence of zeocin and G418 and then retested for luciferase activity (Fig. 4). The cell line, 293B4α consistently and reproducibly had the highest level of luciferase activity and was chosen for further study.

Characterization of the 293B4α cell line

(a) Protein Production

Western blot analysis was used to determine if the 293B4α cell line was producing luciferase, HCV core, HCV serine protease (encoded by the HCV NS3 gene), and HCV RDRP (encoded by the HCV NS5b). Western blot analysis showed that all 4 proteins were produced in the 293B4α cell line (Fig. 5).

(b) Luciferase RNA Production

Theoretically, the only way for luciferase protein to be produced in 293B4α cells is if there is an RDRP present in the cells to transcribe the antisense luciferase RNA into the sense orientation. RT-PCR was used to determine (1) if antisense luciferase RNA transcription, driven by the SV40 promoter, was taking place, and (2) if the antisense RNA was being transcribed into sense luciferase RNA. Oligos LUCFOR and LUCREV were used in the RT-PCR to determine both of these.

Total cytoplasmic RNA was isolated from 5 x 10^6 293B4α cells using the RNAgents RNA Isolation kit as directed by the manufacturers (Promega Corp., Madison, WI). An aliquot,

which was equivalent to 1/50 of the RNA isolated, was used in each RT-PCR. To determine the presence of the antisense and sense strands of RNA, the RT portion of the reaction was run in the presence of only one of the oligos (i.e. LUCREV to detect the antisense strand and LUCFOR to detect the sense strand). The temperature of the reaction was increased to 95 °C for 5 minutes to heat inactivate the RT enzyme and then the other oligo was added and PCR proceeded as normal.

The cytoplasm of the 293B4α cells contained both species of luciferase RNA, whereas 293FL#9 cells did not contain either species (Fig. 6). Likewise, if the RT step was eliminated from the RT-PCR or if the RNA samples were treated with RNase prior to the RT-PCR, no products were produced indicating that the product detected in the RT-PCR of the RNA of 293B4α cells was from RNA and not DNA contamination. Moreover, treating the RNA samples with DNase prior to RT-PCR had no effect on the quantity of product produced in the RT-PCR.

Example 3

Inhibition of Luciferase Activity by Inhibitors of Luciferase, HCV Serine Protease and IRES-Mediated Translation

Four chemical compounds, two known to inhibit the HCV serine protease, one known to inhibit IRES-mediated translation of the HCV RNA, and one known to inhibit firefly luciferase in the 293B4α cell line, were tested for the ability to reduce the level of firefly luciferase in the 293B4α cell line. Thirty-five millimeter plates were seeded with 5 x 10^5 cells/plate and incubated at 37 °C overnight. Media containing various concentrations of the four chemical compounds were added to the cells. Forty-eight hours after

the addition of compound, the cells were lysed with lysis buffer as described above. Luciferase activity was quantified using the Luciferase Assay System (Promega Corp., Madison, WI) as directed by the manufacturer. All four compounds had the ability to inhibit luciferase activity (Table 2).

Table 2

Inhibition of luciferase activity in the 293B4α cell line

Compound	Inhibitor Class	Inhibition[1]
Cmpd A	HCV Protease	++
Cmpd B	HCV Protease	++
Cmpd C	HCV RDRP	+
Cmpd D	Luciferase	+++
Vehicle (0.3% DMSO)	N/A	--

[1]Key to activity: +++: greater than 75% inhibition; ++: between 75% and 50% inhibition; +: between 49% and 25% inhibition; and --: less than 25% inhibition.

Example 4

Semi High-Throughput Assay for Inhibitors of HCV Replication Using the 293B4α Cell Line

The assay begins by plating 3000 293B4α cells/well in 96-well plates and incubating the cells at 37 °C overnight to allow for attachment of the cells to the bottom of the well. Sixteen to twenty-four hours after plating the cells, various concentrations of compound are added to the wells. Thirty-six to forty-eight hours after the addition of compound, media are removed from the cells and the cells are washed once with cold PBS. The cells are lysed in 25 μl of lysis buffer and the plates are stored at -80 °C. The

lysates are thawed at room temperature and 20 μl of lysate and 100 μl of luciferin substrate are placed into the well of an opaque microtiter plate. Luciferase activity is quantified with a luminometer. The potency of the individual compounds is calculated by linear regression. 293FL#9 cells were electroporated with pMJ050 and were selected in G418 and zeocin. Forty-eight clones were randomly selected and tested for the ability to produce luciferase. The luciferase activity in the twelve clones that were able to produce luciferase, was quantified and is shown in Fig. 4. The 293B4α cell line was selected for further study.

293B4α cells were lysed in lysis buffer and the proteins in the lysates were separated by size on a 4-12% poly-acrylamide gel. The proteins were transferred to nitrocellulose by electrophoresis. Luciferase, HCV core, HCV serine protease, and HCV RDRP were detected by antibodies specific for the individual proteins.

Total cytoplasmic RNA was isolated for 293B4α cells using the RNAgents RNA Isolation kit as directed by the manufacturer (Promega Corp., Madison, WI). RT-PCR and 2% of each RNA sample was used to produce DNA from either the sense or antisense luciferase RNA. C= sense orientation of the luciferase gene (coding); A= antisense orientation of the luciferase gene (non-coding); and A/C= single tube RT-PCR, does not differentiate between the coding and non-coding species of RNA. Plasmid DNA containing the luciferase gene was used as a positive control for the RT-PCR. (Fig. 6)

All references cited within this disclosure are hereby incorporated by reference in their entirety.

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